Journal of Tissue Viability 28 (2019) 94–99

Contents lists available at ScienceDirect

Journal of Tissue Viability

journal homepage: www.elsevier.com/locate/jtv

Wound healing activity of terpinolene and α-phellandrene by attenuating inflammation and oxidative stress *in vitro*

Marcella Malavazi de Christo Scherer[a], Franciane Martins Marques[a], Mariana Moreira Figueira[a], Maria Carolina Oliveira Peisino[a], Elisângela Flávia Pimentel Schmitt[a], Tamara P. Kondratyuk[b], Denise Coutinho Endringer[a], Rodrigo Scherer[a], Marcio Fronza[a,*]

[a] Programa de Pós-Graduação em Ciências Farmacêuticas, Laboratório de Produtos Naturais, Universidade Vila Velha, CEP 29102-920, Brazil
[b] Department of Pharmaceutical Sciences, Daniel K. Inouye College of Pharmacy, University of Hawaii at Hilo, Hilo, HI, 96720, USA

ARTICLE INFO

Keywords:
Inflammation
Scratch assay
Migration
Cytokines
Wound healing

ABSTRACT

This study was undertaken to investigate the *in vitro* wound healing effects and the anti-inflammatory and antioxidant activities of terpinolene and α-phellandrene. The *in vitro* stimulatory effects on the proliferation and migration of fibroblasts were assessed using the scratch assay. The anti-inflammatory activity was evaluated using cell-based assays by investigating their influence on nitric oxide (NO), superoxide anion ($O_2^{\bullet-}$), tumour necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) production and using the TNF-α-induced nuclear factor kappa (NF-κB) assay. Antioxidant activity was determined by the ABTS cation radical scavenging capacity, ferric reducing/antioxidant potential (FRAP), and NO free radical scavenging assays. Terpinolene and α-phellandrene significantly increased the migration and proliferation of fibroblasts and suppressed the pro-inflammatory cytokines IL-6 and TNF-α in a dose-dependent manner. Terpinolene and α-phellandrene at a concentration of 100 μM significantly inhibited NO production (41.3 and 63.8%, respectively) in a macrophage cell-culture-based assay, and resulted in reductions in $O_2^{\bullet-}$ production of 82.1 ± 3.5% and 70.6 ± 4.3%, respectively. Moreover, these monoterpenes were verified to suppress NF-κB activity. In summary, terpinolene and α-phellandrene may contribute to broadening clinical options in the treatment of wounds by attenuating inflammation and oxidative stress *in vitro*.

1. Introduction

As the first line of defence of the body, the human skin has important functions, acting as a physical barrier against trauma, microorganisms and parasites, among other vital functions [1]. Any injury that leads to discontinuity of the skin can be called a wound, and usually, it undergoes healing. The wound healing process aims to restrict the tissue damage and allow the restoration of the integrity and functions of the affected tissues. It can be divided into three overlapping phases, named the inflammatory, proliferative and maturation phases, and involves components of the extracellular matrix, resident cells and leukocytes, as well as lipid mediators and proteins [1–3].

Since ancient times, natural products have been used for the treatment of numerous diseases and illnesses globally, attracting scientific and commercial interests. Presently, they continue to play an important role in the health systems in many developed and developing countries and to represent an important pool for the identification of more effective and lower cost therapeutic approaches or novel drug leads [4–6]. Terpenoids represent the oldest and most diverse class of secondary metabolites formed from five-carbon isoprene units called isoprenoids. They represent a highly diversified group of naturally occurring organic compounds, and more than 30,000 different natural terpene metabolites were identified [7]. In plants, terpenoids have a multitude of ecological and physiological functions. They chemically defend against insects and environmental stress and are involved in the repair mechanism of wounds and injuries [2,8].

Recently, continuing our investigations on natural compounds we conducted a screening study focusing on 33 substances including monoterpenes, sesquiterpenes and diterpenes in several actual biological assays related to different stages of the wound healing processes. Among the studied terpenes, our results suggest that the monoterpenes may be considered promising agents for treatment of skin lesions (unpublished results). Monoterpenes are a class of terpenes with a core of 10 carbons cyclized and oxidized through various processes. They

* Corresponding author. Programa de Pós-Graduação em Ciências Farmacêuticas, Laboratório de Produtos Naturais, Universidade Vila Velha - UVV, Av. Comissário José Dantas de Melo, n°21, Boa Vista, Vila Velha, ES, 29102-920, Brazil.
E-mail address: marcio.fronza@uvv.br (M. Fronza).

https://doi.org/10.1016/j.jtv.2019.02.003
Received 8 October 2018; Received in revised form 13 February 2019; Accepted 13 February 2019

constitute 90% of essential oils and are known to exhibit several biological activities, such as anti-inflammatory, antioxidant, antibacterial, wound healing and analgesic effects [8–10]. Although there is a growing interest in understanding the mechanisms underlying the pharmacological activity of monoterpenes, little is known regarding the biological effects of terpinolene and α-phellandrene. Terpinolene has demonstrated non-genotoxic and antioxidant activity [11,12], analgesic and anti-inflammatory properties [13], and anticancer activity through its antiproliferative effects on brain tumour cells [14]. α-Phellandrene has been shown to attenuate the inflammatory response through neutrophil migration inhibition, mast cell degranulation [15] and inhibition of nitric oxide (NO) production in macrophages [16] and through antinociceptive effects [17] and anticancer activity by inducing cell cycle arrest and apoptosis in WEHI-3 cells *in vitro* [18].

The use of techniques and products in wound care associated with substances with anti-inflammatory and antioxidant properties represents a powerful strategy in the treatment of skin lesions. Although there are several therapeutic options available for use that aid the healing process in the treatment of wounds, the identification of more effective and lower cost therapeutic approaches continues to be of interest. Therefore, this study aimed to investigate the *in vitro* wound healing effects of terpinolene and α-phellandrene using various chemical and cell-based assays.

2. Material and methods

2.1. Chemicals and biochemicals

Terpinolene, α-phellandrene, platelet derived growth factor-BB (PDGF), ProLong Gold antifade reagent with 4′,6-diamino-2-phenylindole (DAPI), lipopolysaccharide (LPS), NG-methyl-L-arginine acetate salt (L-NMMA) and nitroblue tetrazolium (NBT) were purchased from Sigma Aldrich® Chemical Co., St. Louis, MO, USA. IL-6 and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were obtained from eBioscience, San Diego, CA, USA. The Dual-Luciferase® Reporter Assay System 10-Pack was obtained from Promega Corporation, USA. All other reagents and solvents used were of analytical grade and were obtained from various commercial sources.

2.2. Antioxidant activity

2.2.1. Ferric reducing/antioxidant power (FRAP) assay

The total antioxidant capacity of terpinolene and α-phellandrene was determined by the ferric reducing/antioxidant power assay as previously described [19,20]. The experiments were carried out at least in triplicate, and the results were expressed as IC_{50} values (μg mL^{-1}).

2.2.2. Determination of the $(NO)^{\cdot}$ scavenging activity

Nitric oxide (NO) was generated from the spontaneous decomposition of sodium nitroprusside (SNP) in phosphate-buffered saline (pH 7.3). Once generated, NO interacts with the oxygen to produce nitrite ions, which were measured by the Griess reaction [20,21]. The optical density was measured at 540 nm using a microplate reader (Multi-Mode Microplate Reader, Filter Max F5, Molecular Devices Spectra, USA). Gallic acid was used as the positive control. The quantification of nitrite was performed by regression analysis from a standard curve of sodium nitrite, and the results were expressed as IC_{50} values (μM).

2.2.3. ABTS cation radical scavenging assay

The antioxidant activity was determined according to Re et al. (1999) [22]. Gallic acid was used as the positive control. The results were expressed as IC_{50} values (μM). The experiments were performed in triplicate at the least.

2.3. Cell lines

Fibroblasts (L929) (ATCC® CCL1TM), RAW 264.7 macrophages (ATCC® TIB-71™) and human embryonic renal cells (HEK 293) transfected with the luciferase-expressing gene (Panomic, Fremont, CA) (retroviruses) were purchased were purchased from the Cell Bank of Rio de Janeiro, Brazil. The cells were cultured in Dulbecco's modified Eagle medium (Sigma Aldrich® Chemical Co., St. Louis, MO, USA) or high-glucose modified culture medium (DMEM-Gibco-BRL Life Biotechnologies, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS-Sigma Aldrich® Chemical Co., St. Louis, MO, USA), 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Sigma Aldrich® Chemical Co, St. Louis, MO, USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4. Assessment of in vitro cytotoxicity

The influence of terpinolene and α-phellandrene on cellular viability was evaluated using the standard colorimetric MTT assay [23]. Briefly, fibroblasts (L929) and RAW 264.7 macrophages were seeded at a density of 6×10^5 cells in 96-well plates and treated with terpinolene and α-phellandrene (1.0–200 μM) for 24 h. Next, the medium was completely removed and100 μL of MTT (1 mg mL^{-1}) was added to each well, and the plate was incubated for 2 h. The formazan crystals formed were dissolved with dimethyl sulfoxide (DMSO), and the optical density was measured at 595 nm using a microplate reader (Multi-Mode Microplate Reader, Filter Max F5, Molecular Devices Spectra, USA). The experiments were performed at least in triplicate.

2.5. In vitro wound healing assay

The activity of terpinolene and α-phellandrene on proliferation and migration of fibroblasts was assessed using the scratch assay [24,25]. Briefly, fibroblasts (1.5×10^6 cells mL^{-1}) were cultured up to confluent cell monolayer. Then, an artificial linear wound was introduced into the monolayers and individually inspected in order to standardize the size of the gap using a microscope connected to a computer. Only gap sizes between 90 and 100 mm wide were used in the experiments. After, cells were exposed to 10, 100 and 200 μM of terpinolene and α-phellandrene to a set of 6 coverslips per dose and incubated for 16 h. PDGF (2 ng mL^{-1}) was used as the positive control. After treatment, the cells were fixed and stained with DAPI. Four representative images from each coverslip of the scratched areas under each condition were photographed at a magnification of 100×. Pictures were captured using a Samsung camera (SDC-415ND) coupled to a Leica microscope (model DMLS) and connected to a computer where the images were stored. Total number of cells into the wounded area were quantified using CellC® software. The results were expressed as the percentage of total cells by comparing the total cell number into the artificial wound of treated group with the total cell number into the untreated control group. Data are representative of three independent experiments.

2.6. Nitric oxide (NO) synthase assay

The nitrite concentration in the culture medium supernatant was measured using the Griess reagent to assess the NO production in LPS-activated RAW 264.7 macrophages [21,26]. Briefly, RAW 264.7 macrophages (2×10^5 cells mL^{-1}) were seeded in 96-well plates and cultured in a humidified incubator with 5% CO_2 at 37 °C for 24 h. Then, the cells were treated with terpinolene and α-phellandrene (1.0–200.0 μM) in phenol-red-free DMEM for 30 min followed by 1 μg mL^{-1} of LPS treatment for 20 h. After the incubation, equal volumes of cell culture supernatant and Griess reagent were combined to measure the NO production. The absorbance was measured in a microplate reader at 540 nm. Nitrite concentration was determined by comparison with a sodium nitrite standard curve. The results were

expressed as the IC_{50} value (μM). Under the same experimental conditions, cellular viability was examined in parallel by the MTT assay.

2.7. Determination of intracellular superoxide anion

The intracellular production of superoxide anion ($O_2^{\bullet-}$) was determined by the nitroblue tetrazolium (NBT) reduction assay as previously described in LPS-activated RAW 264.7 macrophages [27,28]. Briefly, the cells (2×10^5 cell mL^{-1}) were treated with 1 μg mL^{-1} LPS in the presence or absence of terpinolene or α-phellandrene (1.0–200.0 μM) for 24 h. NG-methyl-L-arginine acetate salt (L-NMMA) (500 μM) was used as a positive control. Next, the supernatant was removed, and the cells incubated with 100 μL of NBT (1 mg mL^{-1}) for 2 h. Then, the cells were washed with methanol and dried for 20 min at 37 °C. The formazan crystals formed were dissolved and the optical density was measured at 620 nm. The results are expressed as the percentage of the control without LPS. The experiments were performed at least in triplicate.

2.8. Measurement of cytokines

The culture medium supernatant of LPS-activated RAW 264.7 macrophages after exposure to terpinolene and α-phellandrene (1.0–200.0 μM) for 24 h was used to quantify TNF-α and IL-6 by enzyme-linked immunosorbent assay (ELISA) techniques using specific antibodies (purified and biotinylated) and cytokine standards according to the manufacturer's instructions (eBioscience, San Diego, California, USA). The cytokine levels were expressed in pg mL^{-1}, and the sensitivities were > 10 pg mL^{-1}.

2.9. NF-kB assay

The NF-kB luciferase assay was performed as previously described [26,29]. Human embryonic kidney (HEK) 293 cells (Panomic, Fremont, CA) were seeded into a sterile 96-well plate at 2×10^5 cells per well and grown to approximately 80% confluence by incubating for 48 h. After incubation, the medium was replaced, and the cells were treated with various concentrations (1–100 μM) of the test compounds and incubated for an additional 6 h with or without TNF-α (2 ng mL^{-1}). Next, the cells were washed with PBS, and the luciferase assay was performed using the Luc assay system from Promega (Madison, WI) according to the manufacturer's instructions. The results are presented as the percentage of NF-κB inhibitory activity.

2.10. Statistical analysis

Statistical analyses were performed using GraphPad software (San Diego, CA, 176 USA). Data were expressed as the mean ± standard deviation (SD). Statistical variations were determined using one- or two-way analysis of variance (ANOVA) when appropriate. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Evaluation of the antioxidant activity of terpinolene and α-phellandrene

To assign the antioxidant capacity of terpinolene and α-phellandrene, three analytical methods were used: the ABTS cation radical ($ABTS^{\bullet+}$), the ferric reducing/antioxidant power (FRAP), and the nitric oxide scavenging activity ($NO^{\bullet}$) assays. As presented in Table 1, the NO scavenging activity and ABTS cation radical assays yielded the lowest IC_{50} values, and α-phellandrene showed a better antioxidant capacity with IC_{50} values of 216.9 ± 5.7 and 367.7 ± 1.6 μM, respectively, than terpinolene, which afforded IC_{50} values of 409.4 ± 1.6 and 497.4 ± 14.5 μM, respectively. Terpinolene and α-phellandrene induced a negligible reduction in the complex ferric ion-TPTZ (2,4,6-tri (2-pyridyl)- 1,3,5-triazine) (Table 1).

Table 1
In vitro antioxidant activity of terpinolene and α-phellandrene determined by ABTS, FRAP and NO scavenging assays.

Sample	Antioxidant activity (IC_{50} μM)		
	NO scavenging activity	ABTS	FRAP
Terpinolene	409.4 ± 1.6[a]	497.4 ± 14.5[a]	1325.7 ± 18.4[a]
α-Phellandrene	216.9 ± 5.7[b]	367.7 ± 2.5[b]	1619.6 ± 8.7[a]
Gallic acid	131.2 ± 3.7[c]	12.8 ± 2.1[c]	123.1 ± 1.4[b]

Different letters in the same column correspond to significant differences ($p < 0.05$). Tests (n = 3) were performed in triplicate and expressed as the mean ± standard error.

3.2. Effect of terpinolene and α-phellandrene on cellular viability

Figure S1 reveals that terpinolene and α-phellandrene did not exhibit any cytotoxic effect against L929 fibroblasts and RAW 267.7 macrophages compared to the basal control (cell culture medium alone, considered 100% viability) at up to 200 μM. In contrast, proliferative effects were observed in L929 cells, which exhibited 121.5 ± 3.2% and 119.8 ± 4.1% cellular viability after treatment with 200 μM terpinolene and α-phellandrene, respectively (Figure S1 A and S1B).

3.3. Effect of terpinolene and α-phellandrene on migration and proliferation of L929 fibroblasts

Using the scratch assay, terpinolene and α-phellandrene significantly enhanced the proliferation and migration of fibroblasts compared to the control (untreated cells) in a dose-dependent manner. Fig. 1 A displays representative images of an artificial wound generated on L929 fibroblast cells at 0 h and 16 h post-injury without treatment (control 16 h) and with treatment. Terpinolene and α-phellandrene reached maximum stimulatory effects of 36.3 ± 4.8% and 39.1 ± 3.9%, respectively, at a concentration of 200 μM. PDGF was used as a positive control and exhibited a stimulatory effect of 45.1 ± 5.1% (2 ng mL^{-1}) (Fig. 1B).

3.4. Inhibition of NO production in LPS-induced RAW 264.7 macrophages

Terpinolene and α-phellandrene did not induce changes in the basal NO levels when incubated without LPS (data not shown). As presented in Fig. 2, the nitrite levels in the RAW 264.7 cells exposed to 1 μg mL^{-1} LPS significantly increased by approximately six times compared to those in the negative control cells ($p < 0.05$). However, terpinolene and α-phellandrene significantly suppressed NO production by LPS-stimulated RAW 264.7 cells in a dose-dependent manner. In particular, NO reductions of 41.3 ± 1.4% and 63.8 ± 1.1% were achieved following treatment with 200 μM terpinolene and α-phellandrene, respectively.

3.5. Inhibition of intracellular production of superoxide anion ($O_2^{\bullet-}$)

As presented in Fig. 3, pre-incubation of the RAW 264.7 macrophage cells with terpinolene and α-phellandrene demonstrated a concentration-dependent (1.0–200.0 μM) inhibition of superoxide anion generation in the LPS-stimulated macrophages. Terpinolene and α-phellandrene induced reductions in $O_2^{\bullet-}$ production of 82.1 ± 3.5% and 70.6 ± 4.3% (100 μM) and 82.6 ± 3.5% and 87.6 ± 2.1% (200 μM), respectively.

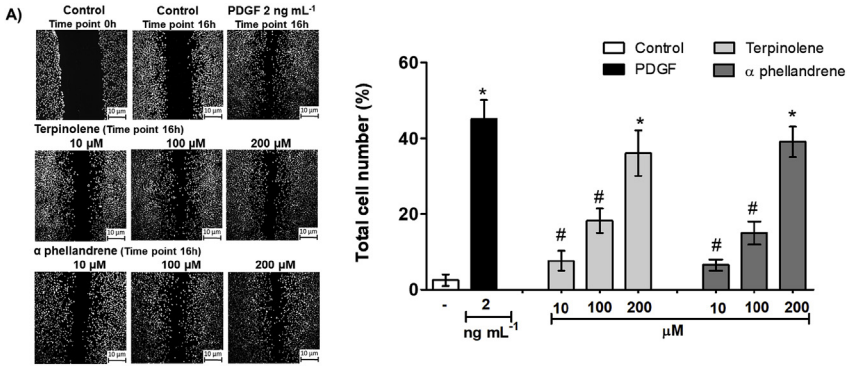

Fig. 1. Effect of terpinolene and α-phellandrene on the proliferative and/or migratory activities of L929 fibroblasts in the scratch assay. **A)** Single representative fluorescence microscope image exhibiting the artificial wound immediately after injury (control 0 h) and after treatments (16 h), as indicated in each panel, at 100×. **B)** Total cell number in the scratch area determined after 16 h of incubation (37 °C, 5% CO_2) in DMEM supplemented with 10% foetal bovine serum. Terpinolene and α-phellandrene were tested at 10, 100, and 200 µM. PDGF 2 ng mL^{-1} was used as a positive control. Bars represent the mean ± SD of three independent experiments. ($p < 0.05$) indicates significant differences compared with the untreated cells (control); # ($p < 0.05$), compared with PDGF.

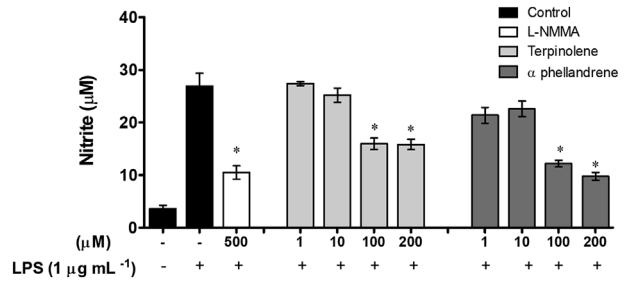

Fig. 2. Inhibitory effects of terpinolene and α-phellandrene on the secretion of nitric oxide by LPS-simulated RAW 264.7 cells. The concentration of nitrite reflects the amount of nitric oxide generated by the RAW 264.7 cells. L-NMMA (500 µM) was used as a positive control. The results are expressed as the mean ± SD of three independent experiments. *($p < 0.05$) indicates significant differences compared with the LPS-treated cells.

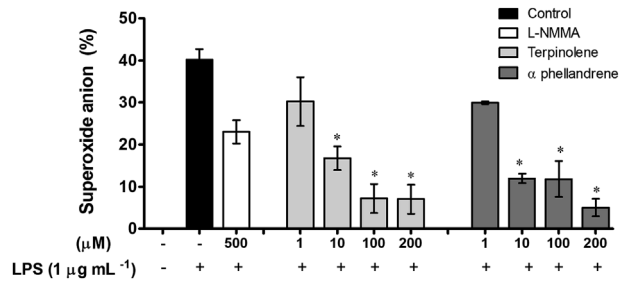

Fig. 3. Inhibitory effects of terpinolene and α-phellandrene on the intracellular production of superoxide anion ($O_2^{\bullet-}$) in LPS-simulated RAW 264.7 cells. The superoxide anion concentration was determined by the nitroblue tetrazolium (NBT) reduction assay. L-NMMA (500 µM) was used as a positive control. The level of superoxide anion in the control cells was arbitrarily expressed as zero. The results are expressed as the mean ± SD of three independent experiments. *($p < 0.05$) indicates significant differences compared with the LPS-treated cells.

3.6. Inhibition of pro-inflammatory cytokine production

Exposure of RAW 264.7 macrophages cells with 1 µg mL^{-1} LPS induced the secretion of IL-6 (Fig. 4A) and TNF-α (Fig. 4B). However, treatment with terpinolene and α-phellandrene significantly reduced the release of IL-6 and TNF-α in a dose-dependent manner ($p < 0.05$).

3.7. Inhibition of TNF-α-induced NF-κB activity

The suppression of NF-κB activation by terpinolene and α-phellandrene was 14.3 ± 2.5% and 26.8 ± 3.1%, respectively, at a concentration of 100 µM (Table S1).

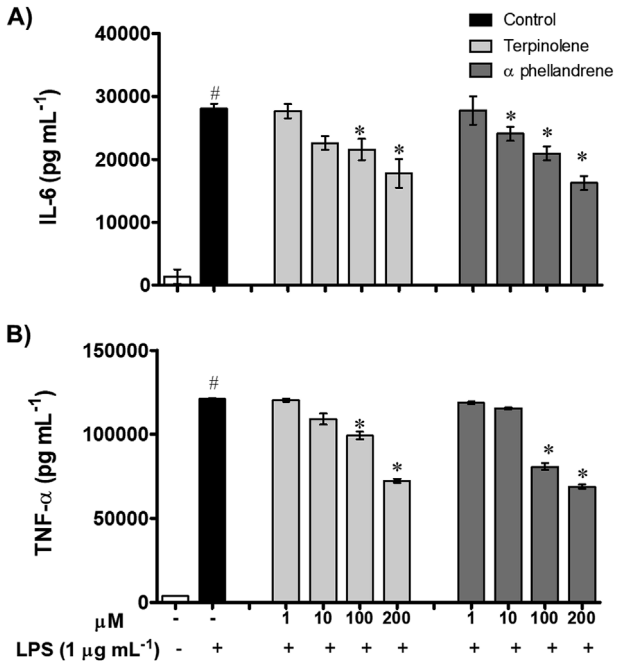

Fig. 4. Effect of terpinolene and α-phellandrene on pro-inflammatory cytokine production in LPS-simulated RAW 264.7 cells. (A) IL-6 and (B) TNF-α production was measured using ELISA kits as described in the material and methods section. The results were expressed as the mean ± SD of two independent experiments. *($p < 0.05$) indicates significant differences compared with the LPS-treated cells; # ($p < 0.05$), compared with the control.

4. Discussion

Wound healing is a complex and well-coordinated process involving different cell types and interactions with soluble mediators, growth factors and cytokines for the repair of injured tissue. Poor wound healing after trauma, surgery, or chronic disease conditions affects millions of people each year and are considered a challenge to healthcare systems globally [1,2,30]. Monoterpenes belong to a large and diverse group of organic compounds with diverse biological activities and therapeutic potential. Despite their importance and the scarcity of studies in this context, terpinolene and α-phellandrene were demonstrated to be promising compounds for the treatment of skin wound conditions.

Dermal fibroblasts are the first line of defence and response to injury and are essential for cutaneous wound repair. After injury, fibroblasts appear in the wound site during the inflammatory phase, proliferate and synthesize growth factors and the new extracellular matrix during the granulation tissue formation, and subsequently generate mechanical forces within the wound to initiate wound contraction [1]. The traditional medicinal plant *Calendula officinalis* was recently approved

for the treatment of minor inflammation of the skin and as an aid in the healing of minor wounds. Among their other biological effects, Calendula extracts stimulated the proliferation and migration of fibroblasts in an *in vitro* wound healing assay, exhibiting similar results as those obtained with terpinolene and α-phellandrene [25,31]. Key to wound healing processes are the proliferation and migration of epithelial cells including fibroblasts, thus they are the basis of *in vitro* studies. The cell culture wound-closure, also known as scratch assay, are widely used in the scientific community [24,25,31]. This experimental model examines the ability of a particular cell line to migrate and subsequently close a wound made in a confluent plate of cells. This test can provide the necessary data that may allow for an understanding of how a particular cell type can spontaneously migrate or respond to a chemo-attractant and directionally migrate toward it. Although the *in vitro* assays are incapable of replicating all the factors involved in complex processes of wound healing, they are considered a valuable *in vitro* tool to gain initial insights into the wound healing potential by providing information on fibroblast proliferation and migration in an artificial wounded area [24,25,31]. Using this valuable *in vitro* tool, terpinolene and α-phellandrene exhibited a significant stimulatory effect on fibroblast proliferation and migration, positively contributing to the cutaneous wound healing process.

Cutaneous injury is known to produce a depression in antioxidant status, as reactive oxygen species (ROS) are produced in response to injury. Although ROS are known to play a positive role in the wound repair process [32], excessive amounts of ROS are deleterious due to their high reactivity [33]. Free radicals including the oxygen atom, as well as reactive molecules such as superoxides and peroxides, are highly unstable molecules and may cause cellular damage via peroxidation of membrane lipids, cross-linking of proteins and breakdown of DNA. Many low-molecular-weight antioxidants, such as vitamins E, terpenes, and phenolic compounds, have been suggested to regulate the redox environment, thereby contributing to healing of skin wounds [33–36]. In this study, we determined that terpinolene and α-phellandrene possess the ability to scavenge reactive species, as evaluated by three different *in vitro* assays. Interestingly, both terpinolene and α-phellandrene markedly decreased the intracellular levels of NO and superoxide anion, as evaluated by cell-based assays, indicating that these compounds may reduce the intracellular oxidative stress environment, contributing to cutaneous wound healing.

The early stage of inflammation is regarded as a critical period in the wound healing process, and pro-inflammatory cytokines have been widely studied because they can regulate the activity of several cells that produce a healing response to tissue injury [37,38]. In this study, it was demonstrated that the production of IL-6 and TNF-α by LPS-simulated macrophages was significantly suppressed by terpinolene and α-phellandrene. Therefore, suppressing the overproduction and activity of these pro-inflammatory cytokines may be successful for the management of skin diseases. Consistent with our findings, α-phellandrene has been recently demonstrated to attenuate the *in vivo* inflammatory response through neutrophil migration inhibition and mast cell degranulation [15].

Although acute inflammation is usually beneficial in the early stage of wound healing, a prolonged acute inflammation phase leads to chronic wounds [1]. The transcription factor NF-κB is considered a pivotal mediator in the human immune system: it regulates the transcription of various inflammatory mediators, e.g., cytokines, chemokines and growth factors [39], which are involved in the inflammatory phase. Thus, inhibiting the NF-κB signalling pathway appears to be an effective therapeutic strategy for the treatment of various inflammatory malignant disorders, including rheumatoid arthritis, atherosclerosis, inflammatory bowel diseases, and wound healing [39]. For monitoring and analyse any cellular response that results in modulation of NF-κB activities, the HEK 293 cell transfected with the luciferase-expressing gene are consider an ideal cellular model [26,29]. Using this cellular model, the NF-κB signalling pathway appeared to be only partially involved in the possible molecular mechanism by which the monoterpenes terpinolene and α-phellandrene inhibit the expression of the pro-inflammatory mediators IL-6, TNF-α, and NO.

5. Conclusion

In summary, the results of the present study showed that terpinolene and α-phellandrene, which share similar chemical characteristics, exhibited similar wound healing properties. Using cell-based assays, both compounds effectively stimulated proliferation and migration of fibroblasts, protected macrophages against cellular oxidative damage, and suppressed the production of pro-inflammatory cytokines (IL-6 and TNF-α) and NF-κB activity. These results provide strong support for the promising candidacy of terpinolene and α-phellandrene for future application in cutaneous wound healing products and formulations.

Conflicts of interest

The authors report no declarations of interest.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. The authors wish to thank the Fundação de Amparo à Pesquisa do Espírito Santo and Conselho Nacional de Desenvolvimento Científico e Tecnológico for the financial support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtv.2019.02.003.

References

[1] Gurtner G, Werner S, Barrandon Y, Longaker M. Wound repair and regeneration. Nature 2008;453:314–21. https://doi.org/10.1038/nature07039.
[2] Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. Wound Repair Regen 2008;16:585–601. https://doi.org/10.1111/j.1524-475X.2008.00410.x.
[3] Schreml S, Szeimies R-M, Prantl L, Landthaler M, Babilas P. Wound healing in the 21st century. J Am Acad Dermatol 2010;63:866–81. https://doi.org/10.1016/j.jaad.2009.10.048.
[4] Atanasov AG, Waltenberger B, Eva-Maria Pferschy-Wenzig TL, Wawrosch C, Uhrin P, Temml V, et al. Discovery and resupply of pharmacologically active plant- derived natural products : a review. Biotechnol Adv 2016;33:1582–614. https://doi.org/10.1016/j.biotechadv.2015.08.001.Discovery.
[5] Harvey AL, Edrada-Ebel R, Quinn RJ. The re-emergence of natural products for drug discovery in the genomics era. Nat Rev Drug Discov 2015;14:111–29. https://doi.org/10.1038/nrd4510.
[6] Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. J Nat Prod 2016;79:629–61. https://doi.org/10.1021/acs.jnatprod.5b01055.
[7] Tholl D. Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. Curr Opin Plant Biol 2006;9:297–304. https://doi.org/10.1016/j.pbi.2006.03.014.
[8] Salminen A, Lehtonen M, Suuronen T, Kaarniranta K, Huuskonen J. Terpenoids: natural inhibitors of NF-KB signaling with anti-inflammatory and anticancer potential. Cell Mol Life Sci 2008;65:2979–99. https://doi.org/10.1007/s00018-008-8103-5.
[9] Barreto RSS, Albuquerque-Júnior RLC, Araújo AAS, Almeida JRGS, Santos MRV, Barreto AS, et al. A systematic review of the wound-healing effects of monoterpenes and iridoid derivatives. Molecules 2014;19:846–62. https://doi.org/10.3390/molecules19010846.
[10] Guimarães AG, Quintans JSS, Quintans-Júnior LJ. Monoterpenes with analgesic activity - a systematic review. Phyther Res 2013;27:1–15. https://doi.org/10.1002/ptr.4686.
[11] Bourgou S, Pichette A, Lavoie S, Marzouk B, Legault J. Terpenoids isolated from Tunisian Nigella sativa L. essential oil with antioxidant activity and the ability to inhibit nitric oxide production. Flavour Fragrance J 2012;27:69–74. https://doi.org/10.1002/ffj.2085.
[12] Turkez H, Aydın E, Geyikoglu F, Cetin D. Genotoxic and oxidative damage potentials in human lymphocytes after exposure to terpinolene in vitro. Cytotechnology 2015;67:409–18. https://doi.org/10.1007/s10616-014-9698-z.
[13] Macedo EMA, Santos WC, Sousa Neto BP, Lopes EM, Piauilino CA, Cunha FVM, et al. Association of terpinolene and diclofenac presents antinociceptive and anti-

inflammatory synergistic effects in a model of chronic inflammation. Braz J Med Biol Res 2016;49. https://doi.org/10.1590/1414-431X20165103. pii: S0100-879X2016000700602.
[14] Aydin E, Türkez H, Taşdemir Ş. Anticancer and antioxidant properties of terpinolene in rat brain cells. Arh Hig Rada Toksikol 2013;64:415–24. https://doi.org/10.2478/10004-1254-64-2013-2365.
[15] Siqueira HDS, Neto BS, Sousa DP, Gomes BS, da Silva FV, Cunha FVM, et al. α-Phellandrene, a cyclic monoterpene, attenuates inflammatory response through neutrophil migration inhibition and mast cell degranulation. Life Sci 2016;160:27–33. https://doi.org/10.1016/j.lfs.2016.07.008.
[16] Cabral C, Poças J, Gonçalves MJ, Cavaleiro C, Cruz MT, Salgueiro L. Ridolfia segetum (L.) Moris (Apiaceae) from Portugal: a source of safe antioxidant and anti-inflammatory essential oil. Ind Crops Prod 2015;65:56–61. https://doi.org/10.1016/j.indcrop.2014.11.041.
[17] Lima DF, Brandã MS, Moura JB, Leitão JMRS, Carvalho FAA, Miúra LMCV, et al. Antinociceptive activity of the monoterpene α-phellandrene in rodents: possible mechanisms of action. J Pharm Pharmacol 2012;64:283–92. https://doi.org/10.1111/j.2042-7158.2011.01401.x.
[18] Lin J, Hsu S, KW L, YS M, CC W, HF L, et al. Alpha-phellandrene-induced apoptosis in mice leukemia WEHI-3 cells in vitro. Environ Toxicol 2016;31:1640–51. https://doi.org/10.1002/tox.22168.
[19] Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 1996;239:70–6. https://doi.org/10.1006/abio.1996.0292.
[20] Bahiense JB, Marques FM, Figueira MM, Vargas TS, Kondratyuk TP, Endringer DC, et al. Potential anti-inflammatory, antioxidant and antimicrobial activities of Sambucus australis. Pharm Biol 2017;55:991–7. https://doi.org/10.1080/13880209.2017.1285324.
[21] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem 1982;126:131–8. https://doi.org/10.1016/0003-2697(82)90118-X.
[22] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999;26:1231–7. https://doi.org/10.1016/S0891-5849(98)00315-3.
[23] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63. https://doi.org/10.1016/0022-1759(83)90303-4.
[24] Liang C-C, Park AY, Guan J-L. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc 2007;2:329–33. https://doi.org/10.1038/nprot.2007.30.
[25] Fronza M, Heinzmann B, Hamburger M, Laufer S, Merfort I. Determination of the wound healing effect of Calendula extracts using the scratch assay with 3T3 fibroblasts. J Ethnopharmacol 2009;126:463–7. https://doi.org/10.1016/j.jep.2009.09.014.
[26] Kondratyuk TP, Park E-J, Yu R, van Breemen RB, Asolkar RN, Murphy BT, et al. Novel marine phenazines as potential cancer chemopreventive and anti-inflammatory agents. Mar Drugs 2012;10:451–64. https://doi.org/10.3390/md10020451.
[27] Choi HS, Jun WK, Cha YN, Kim C. A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. J Immunoassay Immunochem 2006;27:31–44. https://doi.org/10.1080/15321810500403722.
[28] Sarna LK, Wu N, Hwang S-Y, Siow YL, O K. Berberine inhibits NADPH oxidase mediated superoxide anion production in macrophages. Can J Physiol Pharmacol 2010;88:369–78. https://doi.org/10.1139/Y09-136.
[29] Homhual S, Zhang HJ, Bunyapraphatsara N, Kondratyuk TP, Santarsiero BD, Mesecar AD, et al. Bruguiesulfurol, a new sulfur compound from Bruguiera gymnorrhiza. Planta Med 2006;72:255–60. https://doi.org/10.1055/s-2005-873171.
[30] Frykberg RG, Banks J. Challenges in the treatment of chronic wounds. Adv Wound Care 2015;4:560–82. https://doi.org/10.1089/wound.2015.0635.
[31] Nicolaus C, Junghanns S, Hartmann A, Murillo R, Ganzera M, Merfort I. In vitro studies to evaluate the wound healing properties of Calendula officinalis extracts. J Ethnopharmacol 2017;196:94–103. https://doi.org/10.1016/j.jep.2016.12.006.
[32] André-Lévigne D, Modarressi A, Pepper MS, Pittet-Cuénod B. Reactive oxygen species and NOX enzymes are emerging as key players in cutaneous wound repair. Int J Mol Sci 2017;18. https://doi.org/10.3390/ijms18102149. pii: E2149.
[33] Schäfer M, Werner S. Oxidative stress in normal and impaired wound repair. Pharmacol Res 2008;58:165–71. https://doi.org/10.1016/j.phrs.2008.06.004.
[34] Zengin H, Baysal AH. Antibacterial and antioxidant activity of essential oil terpenes against pathogenic and spoilage-forming bacteria and cell structure-activity relationships evaluated by SEM microscopy. Molecules 2014;19:17773–98. https://doi.org/10.3390/molecules191117773.
[35] Badhani B, Sharma N, Kakkar R. Gallic acid: a versatile antioxidant with promising therapeutic and industrial applications. RSC Adv 2015;5:27540–57. https://doi.org/10.1039/c5ra01911g.
[36] de Lima Silva JJ, Pompeu DG, Ximenes NC, Duarte ASG, Gramosa NV, de Morais Carvalho K, et al. Effects of kaurenoic acid and arginine on random skin flap oxidative stress, inflammation, and cytokines in rats. Aesthet Plast Surg 2015;39:971–7. https://doi.org/10.1007/s00266-015-0559-8.
[37] Fullerton JN, Gilroy DW. Resolution of inflammation: a new therapeutic frontier. Nat Rev Drug Discov 2016;15:551–67. https://doi.org/10.1038/nrd.2016.39.
[38] Lai Y, Dong C. Therapeutic antibodies that target inflammatory cytokines in autoimmune diseases. Int Immunol 2016;28:181–8. https://doi.org/10.1093/intimm/dxv063.
[39] Park M, Hong J. Roles of NF-κB in cancer and inflammatory diseases and their therapeutic approaches. Cells 2016;5. https://doi.org/10.3390/cells5020015. pii: E15.